NUTRITIONAL REQUIREMENTS OF THE EARLY CHICK EMBRYO. III. THE METABOLIC BASIS OF MORPHOGENESIS AND DIFFERENTIATION AS REVEALED BY THE USE OF INHIBITORS [1]

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Introduction

In an attempt to learn something about the metabolic changes underlying the developmental processes of morphogenesis and histogenesis, a study of the nutritional requirements of the embryo was begun several years ago (Spratt, 1947). It was thought that a better understanding of the control mechanisms which characterize the differentiation process could be obtained by an examination and comparison of the nutritional properties of differentiating cells with "undifferentiated" and already differentiated cells. Subsequent studies have shown that both initiation and maintenance of particular cellular movements and differentiations of the early chick embryo are dependent upon an adequate nutrient environment, and that different developmental activities have both quantitatively and qualitatively different environmental requirements (Spratt, 1948a, 1948b, 1949a, and 1950). Furthermore, it was shown that differences in metabolism of the completed organs and tissues of the adult are already present during their development.

A further analysis of the biochemical reactions underlying the various developmental activities has been attempted by the use of metabolic inhibitors, substances which have been shown to block or interfere with enzyme activity. Differences in the effects of inhibitors on different embryonic processes would, presumably, indicate differences in the metabolic pathways being used.

Correlation of the effects of inhibitors with those produced by several other environmental changes on particular developmental processes may enable us to construct a better over-all picture of the metabolic basis of embryonic development, even though many important details are lacking. Evidence to be presented, coupled with that provided by preceeding studies, enables us to make the following tentative generalization.

Regions of the chick embryo where cells are undergoing active and rapid differentiation (*e.g.*, the node, the fore-brain, etc.) are more dependent upon an adequate nutrient environment than are regions already differentiated or "undifferentiated." In other words, the pattern of metabolic (enzymatic) activity seems to be directly related to the pattern of differentiation activity.

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Experimental Methods

Approximately 600 chick blastoderms (definitive primitive streak to 8-somite stages) were removed from incubated eggs and explanted to the surface of synthetic media *in vitro*. Operative techniques, culture methods and methods of preparing media were the same as those previously described (Spratt, 1947, 1948a and 1949a). Compositions of the various control and experimental media will be given below. Results have been recorded in the form of camera lucida drawings of the living explants, made by the author. Most of the explants were dissected under saline; a few were fixed, stained and sectioned for study of histological details.

Procedure and Results

In studying the effects of inhibitors on early development, two types of control series were set up for each experiment: (1) embryos explanted to non-nutrient, Ringer media and (2) embryos explanted to glucose-Ringer media. An additional type of control series was set up for each experiment on the effects of monoiodoacetate and sodium fluoride: (3) embryos explanted to sodium pyruvate-Ringer media.

A. Development on Control Media

(1) Non-nutrient, Ringer medium. The medium has the following composition, expressed in grams per 100 ml. of medium: NaCl 0.9, KCl 0.042, $CaCl_2$ 0.024, $Na_2HPO_4 \cdot 12H_2O$ 0.0145, KH_2PO_4 0.0026, $NaHCO_3$ 0.055, Agar 0.35.

Explants to the non-nutrient medium begin to degenerate after an initial lag period (2–4 hrs.). The characteristic feature of the degenerative process is a breakdown of structural organization at the organ and tissue levels and dispersal of the cells (Spratt, 1947, 1948a and 1949a). The spatial pattern and time course of the degenerative process is illustrated in Table I. The region most sensitive to extreme starvation is the node, the center of most active morphogenesis and histogenesis during the first two days' development. A second region of high susceptibility is the anterior end of the embryonic axis, the head-fold, where foregut, fore-brain and optic vesicle formation occur.

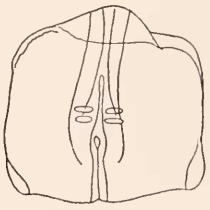
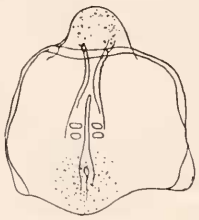
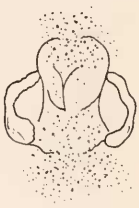
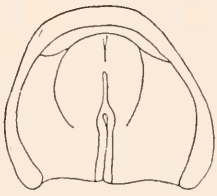
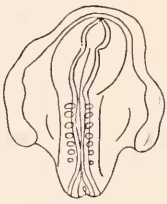
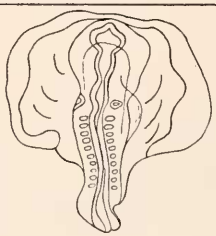
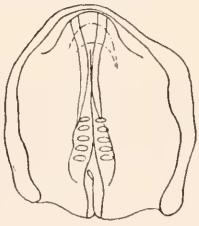
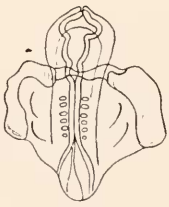
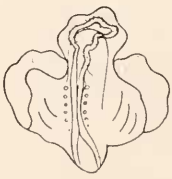
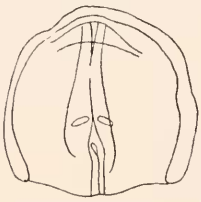
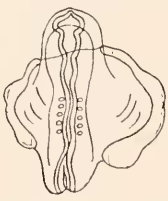
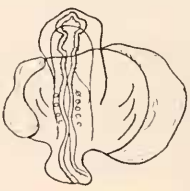
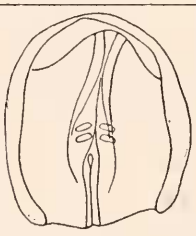
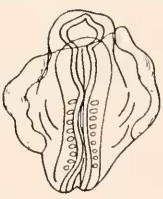
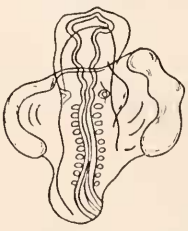
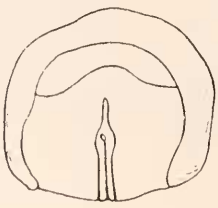
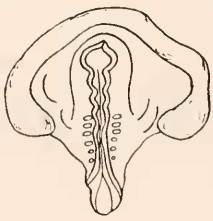
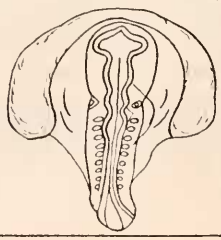
(2) Glucose-Ringer medium. This is simply the non-nutrient medium containing 10^{-3} to $5 \times 10^{-2}M$ d-glucose. In most instances, $10^{-2}M$ glucose was used.

Explants to the glucose medium undergo morphogenesis and differentiation as illustrated by the typical example in Table I: (a) regression of the primitive streak, formation and elongation of the notochord, (b) formation of the head-fold and neural plate, (c) development of the brain, spinal cord, optic vesicles, otocysts and somites, (d) formation and pulsation of the heart, etc. In the absence of an exogenous nitrogen source, the extent of histogenesis is inferior to that obtained in embryos explanted to yolk-albumen media (Spratt, 1947).

The effects of partial carbohydrate starvation (accomplished by decreasing the glucose concentration or by using a less efficiently utilized sugar) were again observed. The results confirm those previously reported (Spratt, 1949a and 1950) in showing that the embryonic region most sensitive to the nutritionally poor environment is the node, with the head-fold region the next most sensitive.

TABLE I

Control series

MEDIUM	NO. EXPL	STAGE EXPLANTED	GENERALIZED RESULTS	
			$10^{\pm}$ HOURS IN VITRO	$20^{\pm}$ HOURS IN VITRO
RINGER-BUFFER	56			
RINGER-BUFFER GLUCOSE 10^{-3}–5×10^{-2}M	140			
RINGER-BUFFER PYRUVATE 10^{-2}–4×10^{-2}M	105			
RINGER-BUFFER LACTATE 2×10^{-2}M	32			
RINGER-BUFFER PYRUVATE 2×10^{-2}M + GLUCOSE 10^{-3}M	28			
RINGER-BUFFER PYRUVATE 2×10^{-2}M + GLUCOSE 2.5×10^{-4}M	4			

(3) Pyruvate-Ringer medium. The non-nutrient medium containing 10^{-2} to $4 \times 10^{-2} M$ sodium pyruvate. In most instances, $2 \times 10^{-2} M$ was the concentration used.

The type of development which occurs on the pyruvate control medium is illustrated in Table I. Although pyruvate can be utilized for supporting some of the early developmental changes, it is inferior to glucose (Spratt, 1949b and 1950). After the first 15–20 hours' cultivation practically all further development ceases and the embryos shrink to an appreciably smaller size than the corresponding glucose controls. Histogenesis of the brain and heart is also inferior. Development on sodium lactate media ($2 \times 10^{-2} M$) is approximately equivalent to that on pyruvate. Some observations indicate that lactate is slightly superior (Table I) but this needs further study.

B. Development on Media Containing Combinations of Glucose and Pyruvate or Lactate

Seven experiments in which a sub-minimal amount of glucose ($2.5 \times 10^{-4} M$) was added to either a minimal ($5 \times 10^{-3} M$) or optimal ($2 \times 10^{-2} M$) pyruvate medium were done.[3]

Results of two experiments are shown in Table I. Here both minmial ($10^{-3} M$) and sub-minimal ($2.5 \times 10^{-4} M$) amounts of glucose plus $2 \times 10^{-2} M$ pyruvate result in development indistinguishable from that of the glucose (10^{-3} to $10^{-2} M$) controls. In general, when both glucose and pyruvate (even at molar concentration ratios of 1 :20 or 1 :80, respectively) are present in the medium, the embryos are similar in their extent of development to those utilizing glucose alone—not dwarfed like the pyruvate controls. Combinations of sodium lactate and glucose have yielded similar results.

C. Metabolic Inhibitor Effects

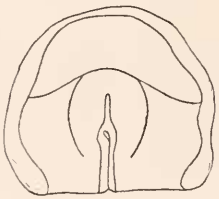
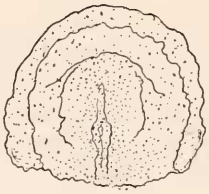
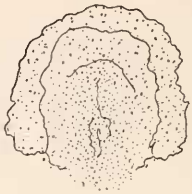
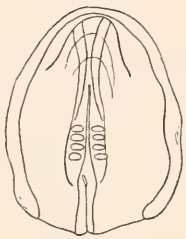
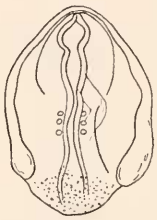
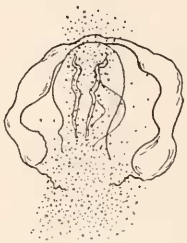
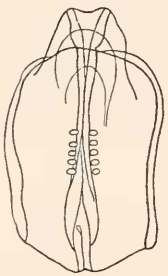
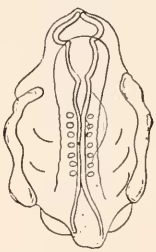
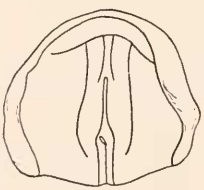
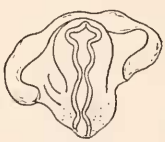
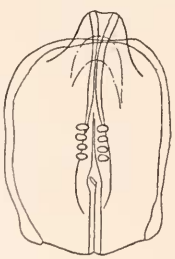
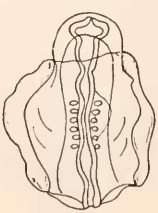
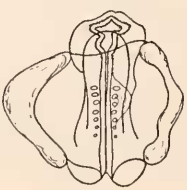
(1) Monoiodoacetate (sodium salt). In these and all other inhibitor experiments, a measured amount of a sterilized stock solution of the inhibitor was added to the medium after it had been autoclaved and cooled to about 40° C. The pH of the medium was adjusted to 7.5–8.5 with NaOH (8.0 in most experiments). The pH was measured with the Beckman pH meter at the beginning and end of each experiment and found to remain constant in almost all instances.

The results obtained are summarized in Table II. It is to be noted that $10^{-4} M$ iodoacetate produces almost immediate stoppage of development and complete degeneration, dispersal and disintegration of the entire embryo. The degree of inhibition at this concentration compared with the type of degeneration resulting from substrate deprivation (non-nutrient controls), indicates that the utilization of endogenous reserves as well as of exogenous substrates is blocked. At lower inhibitor concentrations ($5 \times 10^{-5} M$ and $2 \times 10^{-5} M$) the effects are less drastic and become differential (Table II). It is noteworthy that the time course and

[3] It is possible that the utilization of an excess amount of pyruvate might "throw out of gear" the entire metabolic mechanism by leading to the accumulation of excess endogenous metabolites such as ATP. Glucose might act as a phosphate acceptor and prevent its accumulation (Ochoa, 1941).

Table II

Monoiodoacetate

MEDIUM	NO. EXPL	STAGE EXPLANTED	GENERALIZED RESULTS	
			10± HOURS IN VITRO	20± HOURS IN VITRO
GLUCOSE 10^{-2}M + CH_2ICOOH 10^{-4}M	10			
GLUCOSE 10^{-2}M + CH_2ICOOH 5×10^{-5}M	26			
GLUCOSE 10^{-2}M + CH_2ICOOH 2×10^{-5}M	26			
PYRUVATE 2×10^{-2}M + CH_2ICOOH 10^{-4}M	9			
PYRUVATE 2×10^{-2}M + CH_2ICOOH 5×10^{-5}M	17			

spatial pattern of the differential degenerative process is almost identical with that occurring in the non-nutrient controls: the node and the head-fold, in this order, being the two most sensitive regions. In other words, actively differentiating regions seem to be particularly sensitive to the inhibitor. Eventually the entire neural axis (brain and cord) undergoes degeneration whereas the heart is relatively unaffected, continuing to develop and pulsate (Spratt, 1949a). The fact that the heart will develop in primitive streak blastoderms, in the presence of the inhibitor, argues against the possibility that the differential effect reflects primarily differences in the extent of development of the heart compared with the brain, *i.e.*, not only the maintenance but the initiation of heart-formation is more resistant than the brain. These differential effects on the processes of brain and heart-formation revealed by using the lower concentrations of iodoacetate suggest that the underlying metabolic processes are different.

Further elucidation of the apparently different metabolic mechanisms underlying brain-formation as compared with heart-formation would depend, partly, upon evidence as to the point of action of iodoacetate on the metabolic pathway.[4] The fact that both complete and differential inhibition of development by iodoacetate can be prevented by substitution of sodium pyruvate for glucose in the culture medium (Table II) suggests that the site of action of the inhibitor in the chick embryo is similar to that described for other organisms, namely, that it interferes with triosephosphate dehydrogenase. Partial protection against the effects of $10^{-4}M$ iodoacetate and complete protection against $5 \times 10^{-5}M$ iodoacetate are obtained with $2 \times 10^{-2}M$ pyruvate. The more extensive degeneration of the developing nervous system in the presence of glucose and iodoacetate (which presumably interferes with the important oxidative-phosphorylation reaction) [4] suggests its greater dependence upon oxidative metabolism as compared with the heart. Partial prevention, with pyruvate, of the complete degeneration produced by $10^{-4}M$ iodoacetate exhibits a differential pattern of degeneration which is correlated with the regional pattern of developmental activity (Table II).

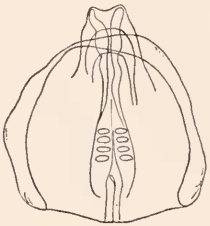
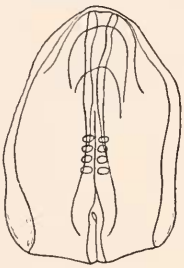
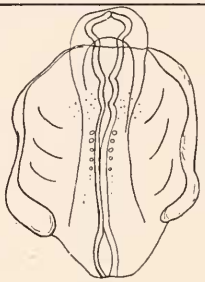
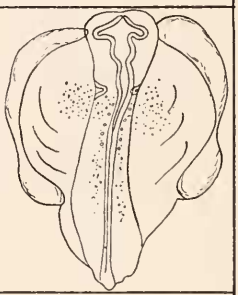
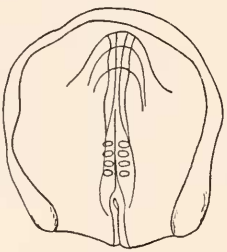
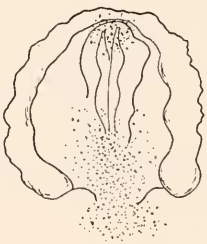
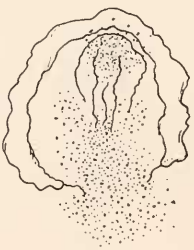
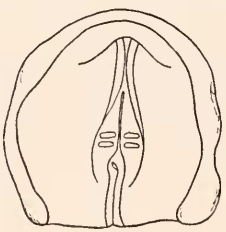
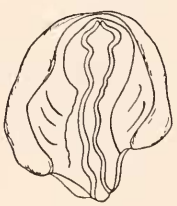
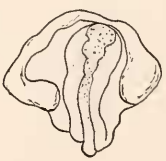
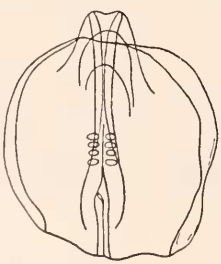
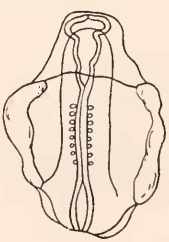
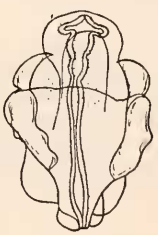
(*2*) *Sodium fluoride.* The effects of this inhibitor and their prevention by substitution of pyruvate for glucose are summarized in Table III. Explants to a glucose-Ringer medium containing $10^{-2}M$ sodium fluoride undergo differential and then complete degeneration, the regional pattern of which is comparable to that resulting from substrate deprivation (Tables I and III). This is in contrast to $10^{-4}M$ iodoacetate which produces immediate and complete degeneration (Table II). Fluoride, at this concentration, apparently does not extensively inhibit the utilization of endogenous reserves.

Lower concentrations of fluoride ($5 \times 10^{-3}M$) result in a differential inhibition of developmental processes which is almost the converse of that obtained with iodoacetate: The heart fails to develop or degenerates if already forming at the

[4] It is generally held that small amounts of iodoacetate (3×10^{-4} to $3 \times 10^{-3}M$) inhibit triosephosphate dehydrogenase, the enzyme catalyzing the reaction: 1, 3 Diphosphoglyceraldehyde $\rightleftarrows$ 1,3 Diphosphoglyceric acid (Dixon, 1937; Adler, v. Euler and Günther, 1938; Rapkine, 1933 and 1938). Originally it was thought that iodoacetate completely blocked anaerobic glycolysis in concentrations which had little or no effect on respiration (Lunsgard, 1930) but subsequently it has been shown that these concentrations ($3 \times 10^{-4}M$) also markedly depress respiration in rat brain and testis (Krebs, 1931; Fuhrman and Field, 1943).

TABLE III

Sodium fluoride

MEDIUM	NO. EXPL	STAGE EXPLANTED	GENERALIZED RESULTS	
			10 ± HOURS IN VITRO	20 ± HOURS IN VITRO
GLUCOSE 10^{-2}M + FLUORIDE 10^{-2}M	37			
GLUCOSE 10^{-2}M + FLUORIDE 5×10^{-3}M	44			
PYRUVATE 2×10^{-2}M + FLUORIDE 2×10^{-2}M	8			
PYRUVATE 10^{-2}M + FLUORIDE 10^{-2}M	7			
PYRUVATE 2×10^{-2}M + FLUORIDE 10^{-2}M	16			

time of explantation, whereas the central nervous system continues its development and shows little if any harmful effect of the fluoride. At lower fluoride concentrations (2.5×10^{-3} and $10^{-3}M$) there is progressively less effect upon heart-formation. The typical picture of the differential effect is that of an apparently normal embryo with a mass of dispersed and degenerating mesodermal cells occupying the position in which the heart would have formed. The degenerative effect of fluoride is not limited to heart mesoderm but involves, to some extent, the somite and lateral plate mesoderm. In a number of cases, posterior development of the neural tube is indirectly inhibited, presumably by the absence of inducing mesoderm. Some of these effects are illustrated in Figure 1.

Interpretation of the differential effects of fluoride, in terms of interference with the metabolic mechanisms underlying heart as compared with brain-formation, is largely dependent upon knowledge of its site of action.[5] The fact that complete inhibition of development by $10^{-2}M$ fluoride can be prevented by substitution of a

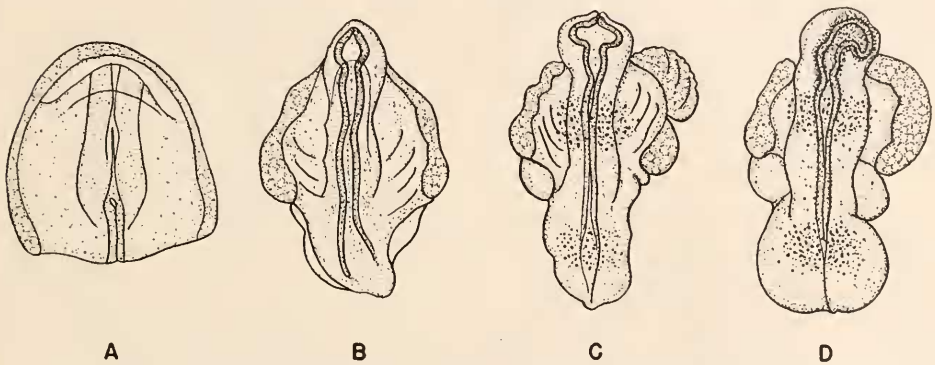


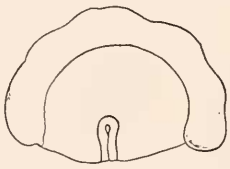
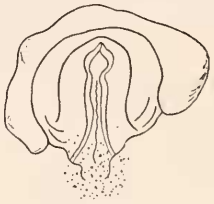
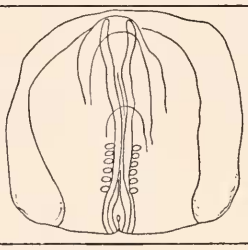
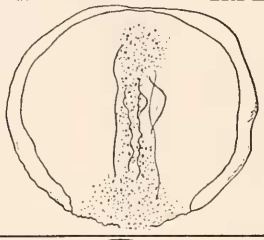
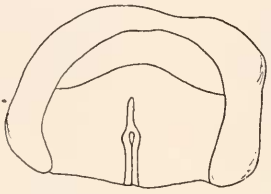
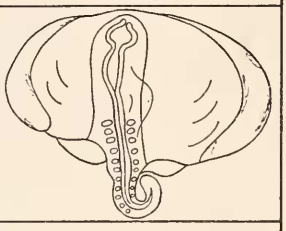
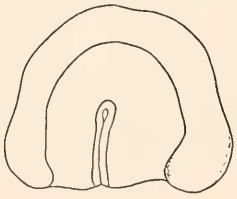
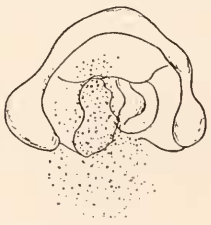
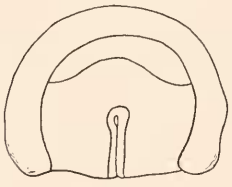
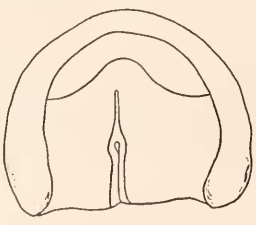
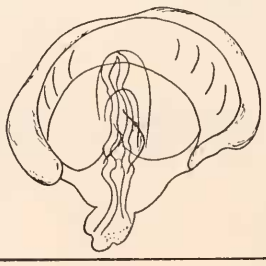
FIGURE 1. Camera lucida drawings of the anterior half of a living explant during its development on a medium containing Ringer, $10^{-2}M$ glucose, and $5 \times 10^{-3}M$ sodium fluoride. A. Stage explanted; B. 8 hours later; C. 18 hours after explantation; D. 38 hours after explantation. Note the degenerating heart mesoderm.

sufficient amount ($2 \times 10^{-2}M$) of pyruvate for glucose (Table III) suggests that the inhibitor is blocking enolase activity. If this is true, the greater sensitivity of the heart as compared with the developing brain would indicate its greater dependence upon a glycolytic energy source. Since the effect of fluoride on respiration is relatively indirect and slight (see Gemmill, 1939, for discussion of the mechanism of fluoride inhibition) compared with the effect of iodoacetate, one would expect that its interference with the metabolic basis of brain-formation would be correspondingly less drastic. Eventually, the piling up of metabolic intermediates in the presence of fluoride would presumably interfere with the glycolytic and oxidative pathways above pyruvate. Indeed, fluoride does have a delayed inhibitory effect upon the nervous system during the second day of cultivation (Fig. 1).

[5] It is generally held that fluoride inhibits the enzyme enolase which catalyzes the glycolytic reaction: 2-Phosphoglyceric acid $\rightleftarrows$ Phosphoenolpyruvic acid, by formation of a magesium-fluoride phosphate complex (Meyerhof and Kiessling, 1935; Warburg and Christian, 1942).

Table IV

Citrate, malonate, cyanide, azide
($10^{-2}M$ glucose present in all media)

INHIBITOR	NO. EXPL	STAGE EXPLANTED	GENERALIZED RESULTS 20± HOURS IN VITRO
CITRATE 5×10^{-3}M	29		
MALONATE 10^{-3} M	38		
CYANIDE 5×10^{-3}M	13		
CYANIDE 10^{-2}M	12		
AZIDE 5×10^{-4}M	6		
AZIDE 10^{-4}M	6		

(3) Sodium citrate. Not only is the early embryo unable to utilize citrate as a carbon source but its utilization of glucose is partially inhibited by 5×10^{-3} to $10^{-2}M$ concentrations of sodium citrate (Table IV). It was thought that citrate would produce an inhibitory effect like that of fluoride because of the formation of relatively insoluble magnesium citrate which would displace magnesium, the normal activator of enolase. However, since there are several other enzymes which depend upon magnesium as an activator, only rather general inhibitory effects could be expected. In fact, citrate produces a pattern of differential inhibition similar to that obtained with iodoacetate, malonate, cyanide and azide; development of the central nervous system is inhibited, but not that of the heart.

(4) Sodium malonate. The effects of $10^{-3}M$ sodium malonate are summarized in Table IV. The differential effects produced by this and by twice this concentration are striking; usually no effect of the inhibitor is observed during the first 20 hours *in vitro*. During the next 20 hour period, the central nervous system degenerates completely. Meanwhile the heart forms and functions. The differential effect of malonate is of the same type as that produced by iodoacetate but is still more pronounced, an interesting observation since malonate is a rather specific inhibitor of succinic dehydrogenase (Quastel and Wooldridge, 1928; Ochoa, 1944) in the cyclophorase system and would not be expected to interfere directly with the glycolysis of glucose by the heart. Iodoacetate would presumably not produce as clear cut a separation of anaerobic glycolysis and respiration (see above).

If the effect of malonate on the developing embryo is the result of its competitive inhibition of succinic dehydrogenase, the addition of succinate to the medium should diminish or prevent the effect. Preliminary experiments in which both $2 \times 10^{-3}M$ malonate and $10^{-3}M$ succinate are present in the medium indicate a partial prevention of the malonate inhibition. Greater concentrations of succinate ($4 \times 10^{-3}M$) are more effective. Preliminary incubation for 6 hours in the presence of glucose and $4 \times 10^{-3}M$ succinate, followed by subculture to a medium containing glucose, malonate ($2 \times 10^{-3}M$) and succinate ($4 \times 10^{-3}M$), results in the best (incomplete) protection against the malonate effect. Attempts to by-pass the inhibited step with sodium fumarate have failed, presumably because exogenous fumarate cannot be utilized by the embryo (Spratt, 1949b and 1950).

(5) Sodium cyanide. Since it had previously been shown that oxygen deficiency has a more pronounced effect on development of the central nervous system than on the heart (Spratt, 1948b and 1950) it was of some interest to study the effect of the respiratory inhibitors, cyanide and azide, both of which according to Keilin and Hartree (1939) act on cytochrome oxidase. Reference to Table IV shows that $5 \times 10^{-3}M$ sodium cyanide in the medium (pH 7.8–8.5) has no visible effect on development. Higher concentrations ($10^{-2}M$) give the typical pattern of differential inhibition as regards the time course and spatial pattern of the degenerative process. As would be expected, the developing brain is more sensitive to cyanide than is the heart. This result is comparable with the effects of cyanide on the squid embryo (Marvel and Fisher, 1948).

(6) Sodium azide. Relatively low concentrations of sodium azide ($5 \times 10^{-4}M$) bring about immediate and complete degeneration and disintegration of the explants

(Table IV). As was the case with monoiodoacetate (see above) this concentration of azide apparently blocks the utilization of endogenous as well as exogenous substrates.[6]

Lower concentrations of azide ($10^{-4}M$) are less toxic and tend to produce a pattern of differential degeneration of the same type as that produced by iodoacetate and malonate, but never as distinct. Indeed, the results to date have shown no clear cut differential effects of azide: concentrations which are sufficiently high to bring about degeneration of the central nervous system also seem to inhibit heart-formation; those just low enough to permit heart development result in abnormal but not inhibited brain development (Table IV).

The absence of distinct differential effects of sodium azide is interesting in the light of its action as an inhibitor of synthetic processes (Clifton, 1937; Meyerhof, 1945). In the frog, azide (in contrast to cyanide) also brings about an immediate cessation of development at all stages studied (Spiegelman and Moog, 1945). Whereas some developmental changes, *e.g.*, heart-formation, can occur under partial anaerobiosis (low oxygen tension or cyanide) these cannot occur in the presence of azide, apparently, because of its additional effect upon the coupling between energy yielding and energy requiring reactions. Evidence for such an action of azide is found in the studies of Barth and Jaeger (1947) on phosphorylation in the frog's egg.

(7) Interpretation of differential effects of monoiodoacetate and sodium fluoride. The observation that mesodermal derivatives are more sensitive to fluoride than are ectodermal derivatives, together with the converse observation that ectodermal derivatives are more sensitive to monoiodoacetate than are mesodermal derivatives, indicates differences in the metabolic processes underlying the formation and maintenance of the two different germ layer derivatives. Although the exact nature of the biochemical differences is not revealed by the above observations, the following explanation is offered as a working hypothesis:

a. It has been shown that the brain is more sensitive to oxygen deficiency than is the heart (Spratt, 1948b and 1950).

b. The brain is also more sensitive to cyanide and azide, substances which interfer with cytochrome oxidase activity.

c. The brain is more sensitive to iodoacetate but the heart is more sensitive to fluoride.

d. The interpretation that the concentration of iodoacetate used ($2 \times 10^{-5}M$) is partially blocking both anaerobic glycolysis and respiration (Krebs, 1931; Fuhrman and Field, 1943), and the concentration of fluoride used ($5 \times 10^{-3}M$) is primarily inhibiting glycolysis, is consistent with the hypothesis that the brain depends primarily upon oxidative metabolism, the heart upon glycolysis.

[6] The similarity in the effects of these two inhibitors is interesting in view of the evidence (Spiegelman, Kamen and Sussman, 1948) that azide not only inhibits the Warburg-Keilin System but also the coupling mechanism between oxidation and phosphate esterification in the reaction: 3-Phosphoglyceraldehyde $\leftrightarrows$ 1,3-Diphosphoglyceric acid, *i.e.*, the diphosphoglycerate-transphosphorylase system close to the site of action of iodoacetate.

Discussion

Changes in the environment of the explanted, early embryo whether consisting of (a) the presence of metabolic inhibitors, (b) substrate deprivation (Spratt, 1949a and 1950), (c) partial anaerobiosis (Spratt, 1948b), (d) carbon dioxide deficiency (Spratt, 1949c) or (e) low pH of the medium (Spratt, 1950) all lead to a characteristic pattern of differential inhibition of development, the time course and spatial features of which are essentially identical with the axial gradient pattern of physiological activity of the blastoderm (Child, 1925; Hyman, 1927; Rulon, 1935). The common effects of these diverse environmental modifications are summarized diagrammatically in Figure 2 in terms of regional and gradient differences in sensitivity (density of stippling) of A, head-fold and B, 10-somite blastoderms. Centers of developmental activity (morphogenesis and differentiation) at the two stages are indicated by the symbols X and Y, and the specific developmental events

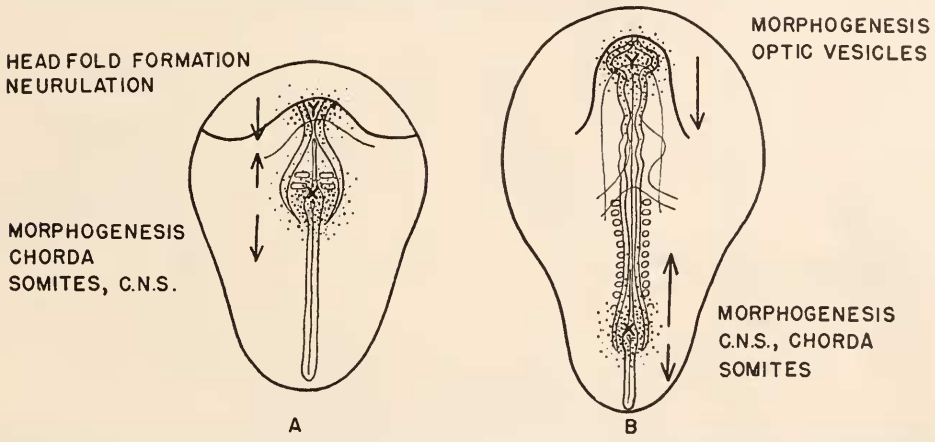


Figure 2. Diagrams summarizing the time course and spatial features of the characteristic pattern of differential inhibition of development produced by inhibitors, starvation, partial anaerobiosis, etc. Note that this pattern is correlated with that of differentiation activity. See text for further descripion.

are noted at the side. The arrows indicate the time course (spread) of the degeneration.

All of the environmental modifications have one primary effect in common: they interfere with the utilization of exogenous substrates upon which the embryo is dependent. In other words, they all retard or inhibit the metabolic processes underlying developmental activities. Furthermore, they all reveal both qualitative and quantitative differences in the nutritional requirements for formation and maintenance of different organs and tissues (Spratt, 1950). On the other hand, each environmental modification, in general, is like the others in demonstrating that the nutritional requirements for the *maintenance* of a differentiated structure (*e.g.*, brain, heart, etc.) are qualitatively the same as those for its *formation* (differentiation). These observations, together with the results of the inhibitor ex-

periments in particular, indicate that there is no qualitative difference between the metabolic basis (at least as regards the fundamental energy-yielding mechanisms) of the differentiation as compared with the maintenance of a structure. The theoretical implications of this concept of the "identity of maintenance and morphogenetic energy" have been discussed by Spiegelman (1945) in connection with his physiological competition hypothesis.

Finally, analysis of the inhibitor and previous experiments on differential nutrient requirements, in so far as they reveal a pattern of both quantitative and qualitative differences in metabolic activity in the early embryo, demonstrates that there is an apparently significant coincidence between the time course and spatial pattern of degeneration produced by inhibitors or starvation and the time course and spatial pattern of differentiation activity (Fig. 2). Regions of the early embryo where differentiation activity is greater (*e.g.*, the node and head-fold, or later, the fore-brain and the segmental plate, etc.) are more sensitive to adverse environmental conditions (presence of metabolic inhibitors,

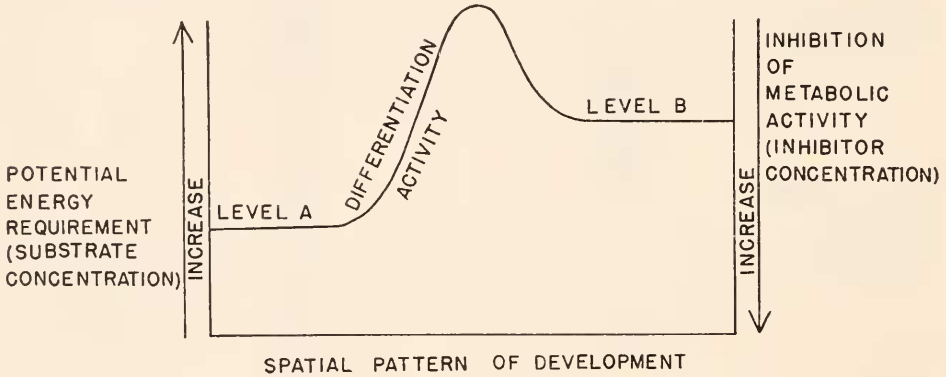


FIGURE 3. Diagram illustrating the relationship of substrate requirements and inhibitor effects to differentiation activity and to a spatial pattern of development. See text for further description.

starvation, oxygen deficiency) than are regions which have already passed through a period of differentiation activity (*e.g.*, hind-brain, spinal cord, somites, pulsating heart, etc.). It is thus not surprising that the energy requirements (in terms of substrate level necessary) for morphogenesis and histogenesis are greater than are those for maintenance. The building up of a new level of structural complexity which characterizes the form-building and tissue-building aspects of differentiation would obviously involve the expenditure of more energy than that needed to maintain the preceeding level of complexity against the ever present tendency toward "structural death."

A more thorough examination of the data pertaining to the differential effects of substrate deprivation, inhibitors, etc. reveals the extremely interesting fact that the energy necessary for the maintenance of an actively differentiating region, transforming from one level of structural complexity to the next, is *more* than the sum of the energy necessary for maintenance of each level. Thus, about 5 mg. glucose, 10 mg. fructose, or 20 mg. galactose (per 100 ml. medium) will maintain relatively

undifferentiated regions; whereas, roughly 10 mg. glucose, 20 mg. fructose or 100 mg. galactose will prevent degeneration of differentiated regions; but 15–20 mg. glucose, 50 mg. fructose or 200 + mg. galactose are necessary to maintain actively differentiating regions (*e.g.*, the node or fore-brain). Although the quantities as stated are only approximations, they are comparatively significant (Spratt, 1949a, pp. 286–291 and 1950). This extra energy requirement may be grossly compared with the "activation-energy" of a chemical reaction except, of course, that there is an *increase* in free energy of the system. In Figure 3 these relations are diagrammatically represented.

For example, as the substrate concentration in the medium is increased it will first satisfy the energy requirements of relatively undifferentiated regions, Level A (*e.g.*, extra-embryonic region compared with embryonic area, spinal cord compared with brain, etc.) and other parts of the blastoderm (both differentiating and differentiated regions) will degenerate and undergo cell dispersal. Further increase would maintain structural Level B, but not the region undergoing the transformation, A → B (*e.g.*, the node). Inhibition of metabolic activity has the converse effect. In terms of synthetic activities, the change from A → B is an increase in rate, surpassing even the rate required to maintain Level B. The greater substrate requirement and sensitivity to inhibitors of an embryonic region undergoing active transformation (*e.g.*, the node) may be compared with its greater differentiation potential [7] and array of tissue-forming potencies. It is tempting to speculate that the structural complexity of such a region is *less* than that of either a relatively undifferentiated or differentiated region.

In conclusion, it would be interesting to interpret the above relations in terms of the underlying enzymatic activities. As an hypothesis, it is suggested that enzymatic activity is inversely proportional to the level of structural differentiation or, conversely, directly proportional to the nutritional (potential energy) requirements of the embryonic region. During the differentiation process (Fig. 3) there would first be an increase in enzyme activity (associated with increased synthesis) followed by a decrease to a new level which, however, is higher than that preceeding the transformation process. The increase followed by a decrease in activity might be proportional to the release from, followed by the incorporation of enzyme proteins into, the structural organization of the cell or cell groups. It is interesting that such rapidly transforming regions as the node and fore-brain also exhibit the greatest capacity for regulation and regeneration (Waddington, 1932; Spratt, 1940). These regions, in contrast to others, might have more "free" enzymes available for the additional catalyses necessary to replace a lost part. Unpublished studies on the reduction of vital dyes, some of the studies of Child and his students, and studies of specific dehydrogenase activities now in progress at least suggest the plausibility of such a concept.

Summary

1. The effects of metabolic inhibitors on the development *in vitro* of approximately 600 chick blastoderms (definitive primitive streak through 8-somite stages) have been studied.

[7] Used in the sense as defined and described by Spiegelman (1945).

2. Embryos explanted to glucose media containing either 10^{-4} to $5 \times 10^{-5}M$ monoiodoacetate or $10^{-2}M$ fluoride rapidly undergo complete degeneration and disintegration. These effects are reversible by substitution of $2 \times 10^{-2}M$ pyruvate (or lactate) for the glucose. At lower inhibitor concentrations of iodoacetate ($2 \times 10^{-5}M$) the central nervous system degenerates or fails to form, but the heart develops and pulsates. Fluoride has almost the opposite effect: concentrations which cause degeneration of the heart ($5 \times 10^{-3}M$) have no appreciable effect upon the developing central nervous system.

3. Other inhibitors: citrate, malonate, cyanide and azide, produce a differential pattern of inhibition similar to that produced by iodoacetate. Azide, however, does not lead to as clear cut a pattern of differential degeneration as do the others.

4. Comparison of the effects of inhibitors with those produced by substrate deprivation, low oxygen tension, etc. reveals that all of these environmental modifications give rise to a characteristic pattern of differential degeneration in the blastoderm, the time course and spatial pattern of which is essentially identical with that occurring in the non-nutrient control series; the node and the head-fold, in this order, being the two most sensitive regions.

5. The pattern of differential inhibition of development produced by the presence of inhibitors or other environmental modifications coincides with the pattern of differentiation activity and, presumably, with the pattern of underlying metabolic (enzymatic) activity.

6. Analysis of the experimental results has led to the formulation of a general hypothesis relating developmental activity, energy requirements and enzyme activity to the gradual transformation from one level of structural complexity to the next.

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